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The major goals of this project are to purify hantavirus proteins (Jonsson) for X-ray structure determination (Arnold). By defining the three-dimensional structures of the N and RDRP proteins of hantaviruses, our studies will afford a means to model drugs that specifically interfere with important stages of viral replication. Particularly, disrupting one or more functions of the RDRP is expected to result in effective disease treatment with little toxicity to host cells. In addition, our research will yield a high through-put in vitro assay for identification of new antiviral drugs. Together, these studies should lead toward effective therapeutic measures for controlling and treating hantaviral infections.

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# Introduction

## Statement of Work

This funded DOD work consists of a collaboration among Dr. Connie Schmaljohn at USAMRIID, Dr. Edward Arnold at the Center for Advanced Biotechnology and Medicine (CABM) at Rutgers University, and Dr. Colleen Jonsson at New Mexico State University (NMSU). The three laboratories cover a wide breadth of expertise in virology (Schmaljohn), biochemistry (Jonsson) and structural biology (Arnold). This report covers all the work although Dr. Schmaljohn received independent funding for the project.

The following milestones are completed (1, 4, 8) or are in progress (2, 5, 7, 9) and will be discussed in the body of the report. We have requested and were granted a no-cost extension to complete the remaining aims.

## Milestones

1. Produce and purify to at least 95% homogeneity the Sin Nombre virus (SNV) nucleocapsid (N) protein (NMSU) - Year 1.

2. Define conditions for crystal production of SNV N protein (CABM) – Year 1.

3. Perform X-ray diffraction analysis of crystals of the SNV N protein to solve the three-dimensional structure (CABM) - Year 2.

4. Produce and purify to at least 95% homogeneity the Hantaan virus (HTNV) RNA dependent RNA polymerase core domain (RDRP) (NMSU) and other soluble portions of the polymerase protein (USAMRIID) - Years 1 and 2.

5. Define conditions for crystal production of HTNV RDRP core domain (CABM) - Year 1.

6. Perform X-ray diffraction analysis of crystals of the HTNV RDRP core domain to solve the three-dimensional structure (CABM) - Year 2.

7. Produce and purify to at least 95% homogeneity the complete HTNV RDRP (NMSU) – Year 2.

8. Complete studies to develop an assay for endonucleolytic cleavage of host mRNA using infectious hantaviruses (USAMRIID) - Year 1.

9. Complete studies to develop an assay for endonucleolytic cleavage of host mRNA using purified, expressed hantavirus polymerase protein (USAMRIID) – Year 2.

## Significance and Rationale



Hantaviruses cause two serious human diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Approximately 150,000 to 200,000 hospitalized cases of HFRS are reported each year throughout the world, with more than half typically occurring in China (Lee, 1996). The rest are found throughout other parts of Asia, Europe, Russia and Scandinavia. Mortality rates for HFRS vary from 1-%-15%, depending in part on which hantavirus caused the infection. HPS is an emerging infectious disease that was first discovered in 1993 in the southwestern United States. Since then, more than 400 cases of HPS have been documented in North and South America, with mortality rates of approximately 40% (Schmaljohn and Hjelle, 1997). Currently, licensed vaccines are not available for the prevention of HFRS or HPS. Therapeutic efforts are generally limited to supportive care, although studies performed in China on HFRS patients suggest that the drug ribavirin provides an improved prognosis when given early in the course of disease. Similar benefits have yet to be documented for HPS patients. No other antiviral drugs for treatment of hantaviral diseases have been identified. The studies described in this proposal are intended to provide a means for the rational design of antivirals for hantaviruses. In addition, because hantaviruses replicate in a manner similar to many other negative strand RNA viruses, our findings may be applicable for the design of effective therapeutics for other viral infections.

As a first step toward our goal of developing antiviral drugs for hantaviruses, we propose to solve the three dimensional structures of the two key proteins needed for hantavirus replication: the RNA dependent RNA polymerase (RDRP) and the nucleocapsid protein (N). Because RDRPs are unique to viruses (i.e., there is no known cellular homologue), this class of enzyme is an attractive target for antiviral agents. The results of the proposed studies will allow us to model drugs that can interact with and disable specific portions of the RDRP. Moreover, the proposed work will generate assays that can be used for rapid screening of large numbers of antiviral drugs. Together, these efforts should lead toward identification of safe and effective means to treat not only known hantaviruses, but also those that are yet to emerge.

# I. Completed Studies

## A. Milestone 1. Produce and purify to at least 95% homogeneity the Sin Nombre virus (SNV) nucleocapsid (N) protein (NMSU)

### A.1. Purification of Recombinant Hantavirus N proteins

The following briefly describes the methods used to purify recombinant N protein (from soluble extracts) that will be used for the proposed studies. This section was more detailed in the first submission. Further details can be found in the Appendix in the manuscript: Jonsson, C.B., Gallegos, J., Fero, P., Xu, X. and C. S. Schmaljohn. Expression, Purification and Characterization of the Sin Nombre Virus Nucleocapsid Protein in Escherichia coli. Protein Express. Purif. 23:134-141

### A.2 Expression and Extraction of the N Proteins of Hantaan and Sin Nombre Viruses

Expression vectors have been constructed that can generate large quantities of the N proteins from Sin Nombre (CC107) and Hantaan viruses (HTNV). The S-segment open reading frames (ORF) were cloned into the *Nde*1 and *Xho*1 sites of pET21b expression vectors (Novagen, Madison, WI), which generate a hexahistidine fusion at the C-terminus. We determined that the hexahistidine tag does not interfere with our RNA binding studies (Severson *et al.*, 1999). Initial studies showed that only a small fraction of the expressed N protein was soluble. Therefore, we optimized the expression strategy and extraction methods of the N protein to provide soluble protein with native extraction buffers. In general, *E. coli* BL21DE3 cells harboring the pET21b-N were grown overnight at 30°C, and then diluted 1:20 in Luria-Bertani (LB) medium containing 200 μg/ml of ampicillin. Following incubation of the diluted culture for 1 h at 30°C, IPTG to a final concentration of 1 mM was added to induce expression of the protein. After 1.5 h, the cells were harvested and resuspended in a solubilization buffer, SB (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 10 mM imidazole), and 0.25 mg/ml lysozyme. Cells were lysed and the soluble material was separated from the insoluble by centrifugation.

### A.3 Purification of the N protein with Nickel NTA chromatography and SP Sepharose Chromatography.

*Nickel Affinity Chromatography.* The soluble portion of the protein extracts was applied to a 1 ml nickel NTA column preequilibrated in SB. The column was washed with SB containing 20 mM imidazole and the N protein was eluted with 250 mM imidazole. We have determined these conditions yield the greatest amount of N protein (7.5 mg/3.2 L). The protein material was dialyzed overnight at 4°C against two changes of 50 mM MES pH 6.2, 200 mM NaCl prior to loading onto a 1 ml Pharmacia SP sepharose column.

*SP Sepharose Chromatography.* Material isolated from soluble fractions were bound to a 1 ml Pharmacia SP sepharose column and a gradient from 200 mM to 1 M NaCl was run by the FPLC (Fig. 1A). The example shown in Fig. 1 is for the SNV CC107 N protein. The two peaks were examined by SDS PAGE (Fig. 1B). The first peak had a very low amount of N protein (Fig. 1B, lanes 1 and 2), and therefore was assayed for its OD260 and OD280 values. The ratio

was 1.7, which strongly suggests the presence of nucleic acids in these fractions. The major peak of N protein from both refolded and soluble preparation eluted at the same time, 9 min, at 660 mM NaCl concentration (Fig. 1A, peak 2, and Fig. 1B, lanes 3 and 4). This suggests that the refolded material has properties similar to the native material. At present, we estimate the isolated N protein has 95% or greater homogeneity.



A. B.

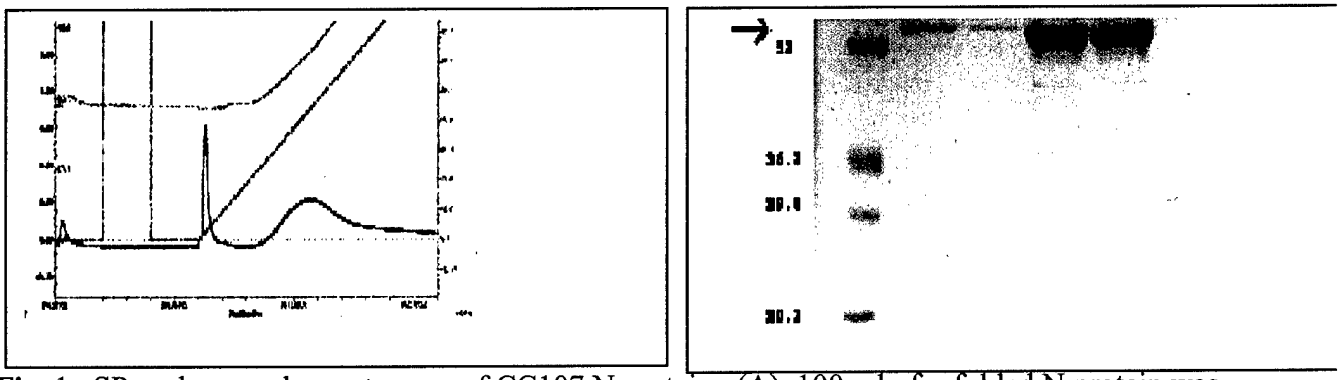

**Fig.** 1. SP sepharose chromatogram of CC107 N protein. (A). 100 ml of refolded N protein was loaded onto a 1 ml Pharmacia SP sepharose column. The column profile shown was run from 200 mM to 1 M NaCl. (B). Fractions from the two major peaks shown in the chromatogram in (A) are presented: Peak 1 -fractions 1 and 2 (Lanes 1 and 2); Peak 2- fractions 5 and 6 (Lanes 3 and 4).

## B. Milestone 4. Produce and purify to at least 95% homogeneity the Hantaan virus (HTNV) RNA dependent RNA polymerase core domain (RDRP) (NMSU) and other soluble portions of the polymerase protein (USAMRIID)

### B.1 Small Scale Expression

We have transformed the pET21b/HTNL into BLR DE3, HMS174 DE3, AD494 DE3, TUNER DE3, Rosetta DE3, and Origami DE3 and performed pilot scale expression studies. Briefly, we have inoculated a 5 ml overnight in LB/Amp200, grown at 37°C and 200 rpm for pilot expression study. Within 12-14 hours, 5 ml of the culture was added to 50 ml 2XYT/Amp100, grow at 30°C at 150 rpm for 1 hour. After 1 hour, 2-1 ml aliquots were taken; centrifuged and resuspended in 70 ul cracking buffer; labeled as uninduced whole cell protein extract. We use the other aliquot to test for protein solubility. The cultures were induced to 1 mM IPTG final concentration. After 1 hour and 3 hours, we took 2-1 ml aliquots and labeled as induced. The other aliquot was extracted into soluble and insoluble fractions. Protein samples were run on each sample on an SDS-PAGE gel (8%bottom-5%stacking); and a duplicate gel was transferred to PVDF membrane using a trans-blot semi dry Bio-Rad cell.

In conclusion the TUNER DE3 cells performed the best in the pilot-scale expression. The RdRp ran consistently between the 150 and 250 MW markers.

### B.2 Optimizing Large Scale Expression

In our attempts to scale up the size of culture, we found that the expression of the RdRp was decreased. We have discovered that we can grow smaller cultures and hence we combine the cell pellets from 6 smaller cultures of 150 ml. This allowed the cells to grow to a higher cell

density, but expression was still not very high and growth of the culture was also inhibited during induction. By comparing the cell pellet of the negative control (no insert—only vector transformed into Tuner cells) to the recombinant cells, the growth inhibition was determined to be caused by leaky expression of the RdRp. Leaky expression was inhibited by the addition of 0.5% glucose to the growth media. The glucose was removed by centrifugation before induction with IPTG. Glucose repression allowed the cells to grow to higher culture densities, so expression of the RdRp was elevated. 

The RdRp was being produced, but was being degraded by proteases. Degradation was detected by western blot analysis of the negative control versus the recombinant cells. Degradation was decreased by growing the cells at 30 °C and inducing at room temperature. Growth and induction at temperatures lower than room temperature had no effect on the stability of the protein. Growing the cells in 2XLB (richer media) also stabilized the protein. Adding protease inhibitor to the extraction buffer also increased stability, but degradation continues to remain a problem.

**Extraction of stable protein**

1. The following buffers have been explored for the extraction of the RdRp:

    1. 50 mM Na phosphate buffer—pH 8.0
       0.3 M NaCl
       20 mM BME
       10 mM CHAPS
       protease inhibitor
    2. 8 M urea
       50 mM Na phosphate buffer—pH 8.0
       0.5 M NaCl
       20 mM BME
       10 mM CHAPS
       0.3 M NaCl
       50 mM Na phosphate buffer—pH 7.5
       10% Glycerol
       1% IGEPAL
       protease inhibitor
    3. 100 mM K Glutamate
       20 mM HEPES—pH 7.6
       1 mM DTT
       1 % IGEPAL
       5-20% glycerol
       protease inhibitor
    4. 0.4 M K Glutamate
       20 mM HEPES—pH 7.6
       1 mM DTT
       1 mM EDTA, no EDTA
       1 % IGEPAL
       5-20% glycerol
       6 M urea, no urea

protease inhibitor



All of these buffers extracted the protein, but the RdRp precipitated in each one immediately after extraction or after one freeze thaw cycle. Also, any attempt to concentrate the protein in these buffers made the protein precipitate out of solution.

2. Extraction followed by dialyzing into these buffers was also explored:

    1. 100 mM HEPES—pH 7.6
       100 mM NaCl
       1 mM DTT
       1 mM EDTA
       10% glycerol
    2. 200 mM K Glutamate
       20 mM HEPES—pH 7.6
       1 mM EDTA
       1 mM DTT
       1% IGEPAL
       20% Glycerol
    3. 20 mM HEPES—pH 7.6
       200 mM K Glutamate
       1 mM DTT
       1 % IGEPAL
    4. 20 mM HEPES—pH 7.6
       200 mM K Glutamate
       1 mM DTT
       1 % Tween 200
    5. 20 mM HEPES—pH 7.6
       200 mM K Glutamate
       1 mM DTT
       1% Triton X
    6. 20 mM HEPES—pH 7.6
       200 mM K Glutamate
       1 mM DTT
       10 mM CHAPS
    7. 20 mM HEPES—pH 7.6
       200 mM K Glutamate
       1 mM DTT
       10% Glycerol

None of these met with success.

Finally, we have made a buffer with 10 mM K glutamate instead of the 100 mM required, and the RdRp was extracted and remained stable. It appears that each of the previous buffers contained too much salt. The following buffer extracts the RdRp in the soluble extract and allows it to remain soluble after freezing and thawing:

10 mM K Glutamate
20 mM HEPES—pH 7.6

1 mM DTT
1 % IGEPAL
20% glycerol
protease inhibitor

**Current Extraction Protocol**

Six 150 ml cultures of 2XLB/Amp 200/0.5% glucose were inoculated with 15 ml of media containing an overnight culture of pET21b/HTNL transformed into Tuner (DE3) cells. These cultures were grown at 30 °C and 220 RPM until the OD600 reached 0.4. The media containing glucose was removed by centrifugation at 5,000 x g for 5 min. The cells were resuspended in an equal amount of 2XLB/Amp 200/1 mM IPTG, and the cultures were induced at 25 °C and 150 RPM for 2.5 h. After induction, the cells were harvested by centrifugation at 5,000 x g for 5 min at 4 °C. The cells were resuspended in 15 ml of Buffer A (150 mM potassium glutamate, 1 x protease inhibitor, 20 mM HEPES-pH 7.6) and centrifuged at 13,000 x g for 10 min. The pellet was resuspended in 15 ml of Buffer B (20 mM HEPES-pH 7.6, 10 mM potassium glutamate, 1 mM DTT, 1 x protease inhibitor, 1 % IGEPAL, 20% glycerol) containing 150 mg of lysozyme. The cells were lysed by douncing for 20 min on ice and sonication twice for 10 s at 40% power. The cell lysate was centrifuged at 13,000 x g for 30 min. The RdRp was extracted in the soluble portion.

**Exploration of Purification Methods**

1. Ni affinity chromatography. All of the RdRp was in the flow-through of the column. We thought this was due to the 6 X histidine tag being buried inside the protein, so I tried adding 2 M, 4 M, and 6 M urea to loosen the structure and make the histidine tag more accessible to the resin. None of these attempts worked, and the RdRp was always collected in the flow through.

2. Dialysis using high MWCO tubing and stirred cell dialysis. Neither the tubing nor the filter paper allowed smaller proteins through. When dialyzed for purification, we used the same buffer for dialysis that I used for extraction. (Buffers listed above)

3. Ammonium sulfate precipitation. Using dry ammonium sulfate, 20%, 40% and 60% ammonium sulfate was added to the soluble extract. After adding the ammonium sulfate, the solution to rested on ice with occasional stirring for 20 min. The solutions were centrifuged at 13,000 x g for 15 min to collect the precipitated proteins. The RdRp precipitated at 20% concentration and easily resuspended in 1 ml of Buffer B. While this did not purify the protein much, it definitely concentrated the RdRp. Trying concentrations of ammonium sulfate that are closer to 20% (10%, 15%, 20% and 25%) may allow more selective precipitation.

4. Size Exclusion Chromatography. Currently, we are exploring concentrating the protein using centriprep devices from Millipore that have a high MWCO and may be used on highly concentrated samples, such as the cell lysate. Since we have found a stable buffer for the RdRp, concentrating the sample should not be as difficult as before. After concentrating (and hopefully clearing smaller proteins from the lysate), we will run the extract over a Sepharose

6 gel filtration column. SEC seems promising, since the RdRp is larger than any of the bacterial proteins.



## C. Milestone 8. Complete studies to develop an assay for endonucleolytic cleavage of host mRNA using infectious hantaviruses (USAMRIID)

### C.1 Development of Enzymatic Assays to Study Components Required for Transcription and Replication

The RdRp has three major enzymatic activities: mRNA synthesis, cRNA synthesis, and vRNA synthesis. Each area can be further subdivided into additional activities. For example, within mRNA synthesis, we expect to observe cap-binding, an endonuclease, and polymerase (initiation, elongation, and termination) activity. The research efforts at USAMRIID focused on establishing methods to monitor the cap-snatching activity and primer-independent transcription.

**Endonuclease Cleavage Assay.** The endonuclease activity of the polymerase provides a relatively simple assay for assessment of the enzymatic activity of the RdRp as opposed to assays calling for full-length vRNA or mRNA synthesis. Due to its' "cap-snatching" function, the RdRp generates small products (15 to 18 nucleotides depending on the substrate) that can be readily visualized by 32P-labeling of the 5′-end of the substrate following separation by denaturing polyacrylamide gel electrophoresis and phosphorimaging/autoradiography. The discrete size of the product, therefore, allows for a simple and direct confirmation of endonuclease activity. Minimally, the components for the development of an endonuclease assay consists of the RdRp, the vRNA template, host mRNA, metal ions, and the appropriate ionic strength and pH. The vRNA/cRNA templates are encapsidated; thus there is may also be a requirement for the N protein. As purified RdRp is not yet available, the above reaction components used purified virions.

(Fig. 2, lane 1). The substrate (sub) is shown with corresponding products (prod) for each reaction. Although much less than seen in the influenza control (Fig. 2, lane 5), a cleavage product was observed. The upper band in lanes 3 and 4 were considered to be the product, while the lower band was not, and could be due to a contaminating nuclease in the virion preparation. Additional work will be focused on characterization of the products and optimizing the yield of product in the assay.

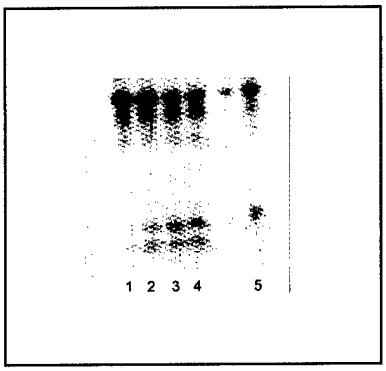


**Fig. 2.** Cap-snatching assay for HTNV.

**Primer-independent polymerase activity of virions**—A rapid membrane assay was developed to characterize the polymerase activity of purified virions. The reaction, performed in a 96-well plate, measures the incorporation of $^{32}$P-UTP into nascent RNA without the addition of template or primer to the reaction. The virion provides the RdRp and the viral genomic RNA template or vRNA. Reactions were terminated and slotted onto DEAE membrane to ascertain the level of new synthesis. As the reaction conditions have not been previously

optimized for any negative strand RdRp from this family *in vitro*, the concentrations of salt and NTP as well as the type and concentration of metal ion were determined. The ionic strength of the polymerase reaction was examined from 40 mM to 140 mM NaCl. Reactions containing 60 and 80 mM NaCl reproducibly showed the highest levels of activity (Fig. 3).

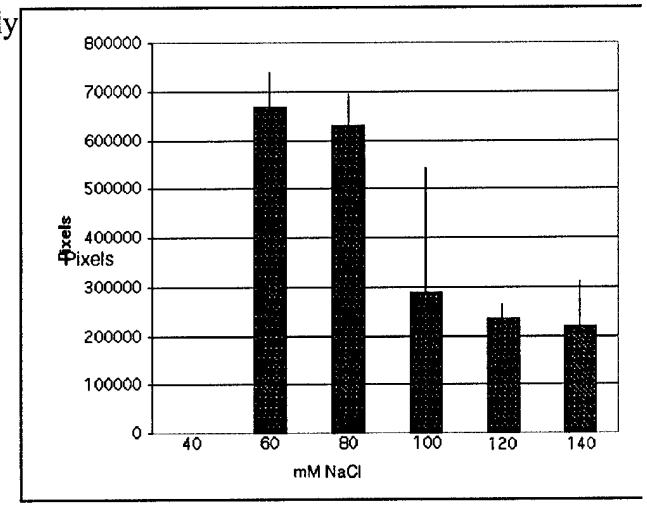


**Fig. 3.** Polymerase activity of virions at different NaCl concentrations.

A key component of viral polymerase reactions is the metal ion (Shapiro and krug, 1988, Plotch et al, 1989). Previously, polymerase activity was demonstrated with a combination of 5 mM $MnCl_2$ and 1.5 mM $MgCl_2$ (Schmaljohn et al, 1983). We examined the presence of $MnCl_2$ in the reaction conditions and $MgCl_2$. Reactions were assembled as described above, however, the final NaCl concentration was 80 mM and metal was omitted. The concentration of $MnCl_2$ was titrated from 0 to 12.5 mM. Typically, reactions containing 7.5 mM $MnCl_2$ showed the greatest level of synthesis (data not shown). Little to no synthesis was observed when reactions were assembled with $MgCl_2$ alone (data not shown). This result is consistent with earlier observations (Schamljohn et al, 1983).

Finally, the concentration of NTP was examined with the membrane assay. In these experiments, reactions were assembled as described above using 80 mM NaCl, 2.5 mM $MgCl_2$ and 2.5 mM $MnCl_2$. The ribonucleotides CTP, ATP, and GTP were omitted from the reaction buffer and titrated at 0.25, 0.50, 0.75 and 1.00 mM (Fig. 3). Of the concentrations tested, reactions conducted at 0.25 mM CTP, ATP and GTP were routinely found to give the highest levels of synthesis. Quantitation of the reactions is presented in Fig 4.

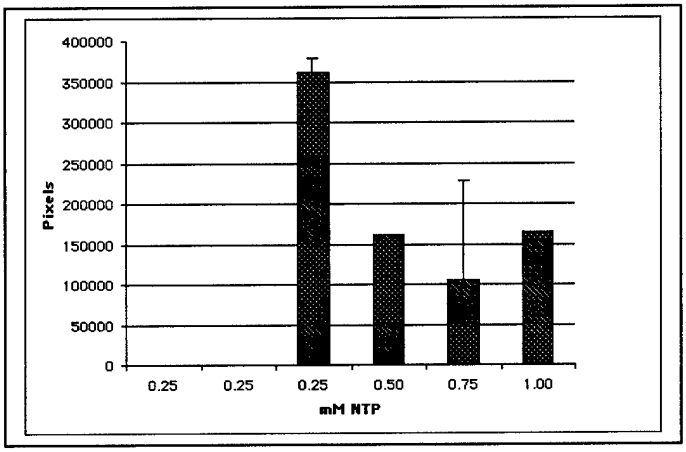


**Fig. 4.** Polymerase activity of virions at different NTP concentrations.

ApG is able to function as a primer in influenza replication reactions (13). Thus, we examined whether the dinucleotide primer could function in our assay system. The 3' end of HTNV genome (3'-AUCAUCAUC) could hypothetically anneal to the ApG at the penultimate U and adjacent C. This could then "slip-back" or prime and realign as proposed by Garcin et al. (1995). Reactions were assembled in the reaction buffer described above. ApG did not increase the level of synthesis observed in two separate experiments (data not shown), and therefore was not examined further.

## II. Ongoing Studies

## A. Studies to define conditions for crystal production and alternative proteins subdomains for crystallization.

### A.1 Define conditions for crystal production of SNV N protein (CABM)

Samples of nucleocapsid protein appear monodisperse by gel filtration, anion exchange chromatography, and gel electrophoresis. The protein can be concentrated to between 10 and 20 mg/ml, which is an appropriate range for crystallization protocols. Limited crystallization experiments have been performed allowing the possible range of crystallization conditions to be narrowed down Crystallization procedures utilize the hanging drop method, by which protein solutions are mixed with appropriate salts and precipitant solutions to induce crystal formation and growth. A trial of a large range of possible conditions has been tested but not crystals have yet formed. It can be difficult to determine appropriate crystallization conditions for protein molecules. Some proteins will crystallize over a wide range of conditions, while others may do so only in a very narrow range. We will continue to utilize commercially available crystallization kits and methodologies. These are very widely used and successful as evidenced in many publications. They have been developed from surveys of successful conditions from thousands of publications, and the crystallization matrices are mathematically determined to cover many variables (such as pH and salt concentration) in a small number of experiments. If these kits are not successful we can screen hundreds of other conditions. Once crystals are grown it is usually straightforward to fine-tune conditions in the attempt to grow large and well-diffracting crystals.

### A. 2 Mapping of the RNA Binding Domain of the Hantavirus N Protein (NMSU)

In order to capitalize on other subdomains that are more soluble, we must first identify the functional regions within the protein. Toward this goal, we have continued previous studies to define the RNA binding domain. To map the location of the RNA binding domain (RBD) of HTNV N protein, fourteen truncated constructs were prepared in the N-terminal, C-terminal or both regions of the N protein (Xu *et al.*, 2002). This work defined a minimal RBD between amino acid residues 175 to 217 that may extend into amino acids 217 to 249.

To further confirm the location of the RNA binding domain as well as provide insight into the use of peptides to explore the RBD, we have recently designed and purchased synthetic peptides of the RBD from Sigma-Genosys (Fig. 5). Dr. Severson has measured the RNA binding levels of each of the peptides with our filter binding methods (Severson et al 1999, Severson et al 2001).

| N | Peptide | | | | | binding |
|---|---|---|---|---|---|---|
| 185-217 | | QSSMKAEEIT | PGRYRTAVCG | LYPAQIKARQ | MIS | wild type |
| 195-217 | | | PGRYRTAVCG | LYPAQIKARQ | MIS | excellent |
| 206-217 | | | | LYPAQIKARQ | MIS | good |
| 175-206 | KHLYVSLPNA | QSSMKAEEIT | PGRYRTAVCG | LYP | | good |
| 175-196 | KHLYVSLPNA | QSSMKAEEIT | PGR | | | weak |
| 175-186 | KHLYVSLPNA | QSS | | | | poor |

**Fig. 5.** Peptides synthesized within the putative HTNV RNA binding domain. A qualitative description of the binding activity is shown adjacent to each substrate.

Previously, we have measured and published the Kd for the full-length HTNVN protein with S-segment viral RNA substrates (53 ± 8 nM) (Severson, 1999). The largest peptide that Sigma-Genosys was able to synthesize was 185-217. Filter binding analysis of this peptide with the 5'end vRNA (1-39) substrate showed it to have a Kd of ~70 nM. ***The culmination of our previous work and these preliminary studies is embodied in this protein-RNA interaction—in that—the macromolecular interaction between these two biomolecules mimics the interaction characterized with the full-length protein and the full-length vRNA substrate.***

## B. Produce and purify to at least 95% homogeneity the complete HTNV RDRP (NMSU).

### B.1 Development of Methods to Overexpress and Purify the HTNV RdRp from E. coli

The open reading frame of the HTNV L-segment was cloned into the bacterial expression vector pET21b, sequenced in both directions, and expressed as a C-terminal hexahistidine fusion protein. At the N-terminal end, a T7 tag was incorporated for visualization by immunoblot. Small scale expression of the RdRp, examined in six bacterial cell lines, was highest in Tuner(DE3) cells. SDS-PAGE revealed a band at 250 kDa, which was confirmed on the corresponding immunoblot using anti-T7 tag antibodies.

Several conditions have been explored for expression and extraction of the RdRp as deiscussed earlier. In the following, our most recent protocol is discussed. A 150 ml culture of LB/Amp100/0.5% glucose was inoculated with a 15 ml overnight culture of Tuner (DE3) *E.coli* cells transformed with the recombinant pET21b/Hantaan L segment plasmid. This culture was allowed to grow at 37 °C and 225 RPM until the culture density reached an optical density of 0.55 at an absorbance of 600 nm. The media was removed by centrifugation at 5,000 x g for 5 min, and the cells were resuspended in 150 ml of LB/Amp 100 containing 1 mM isopropylthiogalactosidase (IPTG). The cells were incubated at 25 °C and 150 RPM for 2.5 h, and then harvested by centrifugation at 5,000 x g for 5 min. The cell pellet was resuspended in 10 ml of 20 mM HEPES (pH 7.6) and 150 mM potassium glutamate. The cells were again harvested by centrifugation, and then resuspended in 10 ml of 20 mM HEPES (pH 7.6), 100 mM potassium glutamate, 5 mM $MgAc_2$, 1 mM DTT, and 1% IPEGAL. The cells were lysed using lysozyme, mechanical lysis, and sonication. The lysate was centrifuged at 13,000 x g for 30 min, and then the pellet was resuspended in 10 ml of the same buffer. The centrifugation was repeated, and the final pellet was resuspended in 10 ml of 0.4 M potassium glutamate, 20 mM HEPES (pH 7.6), 1 mM EDTA, 1 mM DTT, 1% IPEGAL, and 10% glycerol. The centrifugation was repeated, and the pellet was resuspended in 10 ml of 0.4 M potassium glutamate, 20 mM

HEPES (pH 7.6), 1 mM EDTA, 1 mM DTT, 1% IPEGAL, 10% glycerol, and 4 M urea. The mixture was incubated on ice for 15 min and then centrifuged at 13,000 x g for 30 min. The protein extract was incubated with 0.5 ml of Ni-NTA resin for 2.5 h, and then loaded onto a 1 ml column. The column was washed with 5 ml of the extraction buffer containing 15 mM imidazole. The protein was eluted from the column in five 1.5 ml fractions using the extraction buffer containing 250 mM imidazole.



## III. Future studies

### A. Milestone 3. Perform X-ray diffraction analysis of crystals of the SNV N protein to solve the three-dimensional structure (CABM) - Year 2.

Now that effective means of purification have been defined for the N protein, work on milestone 3 will be initiated. We will also use subfragments of the HTNV N protein that were identified to have higher solubility. Further, we have defined a key functional element, the RNA binding domain, within the protein that will be examined as a subdomain with and without its viral RNA substrate.

### B. Milestone 5. Define conditions for crystal production of HTNV RDRP core domain (CABM) and Milestone 6. Perform X-ray diffraction analysis of crystals of the HTNV RDRP core domain to solve the three-dimensional structure (CABM).

The first set of protein products expressed from the RDRP core domain did not yield sufficient amounts for crystallization. The subdomains that were identified for analysis were chosen from alignments of the polymerase with other polymerases with known crystal structure. We are in the progress of defining the core domain via an alternative approach. We will use proteolytic digestion of the full length protein to define the core portion. The major products isolated from the proteolytic digestion will be sequenced. This will define the domains for subsequent subcloning. We did not start with this approach initially because we did not have the full length protein purified.

## Key Research Accomplishments

- Method for purification of soluble, homogenous N protein from E. coli
- Five additional clones for RDRP expression in E. coli
- Expression of three RDRP clones in E. coli
- Purification protocol for RDRP clones in E. coli
- Development of an assay for endonucleolytic cleavage of host mRNA using infectious hantaviruses

- Defined conditions for crystal production of SNV N protein

**Reportable Outcomes**

**Manuscripts**

Jonsson, C.B., Gallegos, J., Fero, P., Severson, W., Xu, X. and C. S. Schmaljohn. (2001) Expression, purification and characterization of the Sin Nombre virus nucleocapsid protein in Escherichia coli. Protein Express. Purif. 23:134-141

Jonsson, C.B., Severson, W., Xu, X, Senutovitch, N., and Claw, S. Critical amino acids in the RNA binding domain of the Hantaan N protein. In preparation.

Leon, L., Thokala, M, and Jonsson, C. Expression of the 246 kDa RNA dependent RNA polymerase in Escherichia coli. In preparation.

Jonsson, C.B. and Schmaljohn, C.S. Primer-independent polymerase activity of the Hantaan virus RNA dependent RNA polymerase. In preparation

**Abstracts**

C. Jonsson. Third International Virus Assembly Meeting, Seeon, Germany, April 1st - 5th 2001.
The nucleocapsid or N protein of hantaviruses encapsidates both viral genomic RNA (vRNA) and the antigenomic RNA (cRNA), but not viral mRNA. Previous work has shown that the N protein has preference for vRNA and this suggested the possibility of a *cis*-acting signal that could be used to initiate encapsidation for the S-segment. To map the *cis*-acting determinants, several deletion RNA derivatives and synthetic oligoribonucleotides were constructed from the S-segment of the Hantaan virus (HTNV) vRNA. N protein-RNA interactions were examined by UV crosslinking, filter binding assays, and gel electrophoresis mobility shift assays to define the ability of each to bind HTNV N protein. The 5′ end of the S-segment vRNA was observed to be necessary and sufficient for the binding reaction. Modeling of the 5′ end of the vRNA revealed a possible stem loop structure (SL) with a large single-stranded loop. We suggest that a specific interaction occurs between the N protein and sequences within this region to initiate encapsidation of the viral genomic RNAs.

American Society of Virology, 20th Annual Meeting, July 2-25, Madison, WI (Poster).
Purification and Characterization of the Catalytic Core Domain of the RNA Dependent RNA Polymerase from Hantaan Virus. Juan-gabriel Gallegos, William Severson, Connie Schmaljohn, Lisa Leon, and Colleen Jonsson. New Mexico State University, Department of Chemistry and Biochemistry. Virology Division, USAMRID.

The Hantaan virus (HTNV) RNA Dependent RNA Polymerase (RdRp) catalyzes the transcription and replication of negative-sense, single-stranded, L, M, and S viral RNAs as well as the transcription of antigenomic or complementary RNAs and messenger RNAs. The RdRp is encoded by the L-segment and has an apparent molecular weight of 246 kDa. Comparing deduced amino acid sequences of the RdRp of HTNV with those of other RNA virus polymerases reveals a region of highly conserved residues between aa 847-1234, which may constitute a catalytic core domain; i.e., a region that is important for catalysis, metal binding, and RNA template-primer interactions. As a first step toward devising an *in vitro* system for studying hantavirus replication, we cloned this predicted domain into the bacterial expression vector pET

21b and expressed it as a hexahistidine fusion protein in BL21 DE3 *E. coli* cells. We purified the expressed polypeptide by nickel affinity and Sepharose cation exchange chromatography and analyzed it by SDS-PAGE and immunoblotting. We detected a polypeptide of the expected size of approximately 44 kDa. We tested the functionality of the core product by using an assay that measures elongation of a primer-template RNA by incorporation of a single $\alpha$-$^{32}$P-ATP. Analysis of reaction mixtures by gel electrophoresis and autoradiography revealed a product one nucleotide longer than the template, thus demonstrating enzymatic functionality of the cloned fragment.



Supported by DOD grant, DAMD 17-00-1-0513.

American Society of Virology, 20th Annual Meeting, July 2-25, Madison, WI (Talk). *Cis*-ACTING SIGNALS IN THE ENCAPSIDATION OF HANTAAN VIRUS S-SEGMENT vRNA BY ITS N PROTEIN.

William E. Severson, Xiaolin Xu, and Colleen B. Jonsson
Graduate Program in Molecular Biology and the Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, NM

The nucleocapsid or N protein encapsidates both viral genomic RNA (vRNA) and the antigenomic RNA (cRNA), but not viral mRNA. Previous work has shown that the N protein has preference for vRNA and this suggested the possibility of a *cis*-acting signal that could be used to initiate encapsidation for the S-segment. To map the *cis*-acting determinants, several deletion RNA derivatives and synthetic oligoribonucleotides were constructed from the S-segment of the Hantaan virus (HTNV) vRNA. N protein-RNA interactions were examined by UV crosslinking, filter binding assays, and gel electrophoresis mobility shift assays to define the ability of each to bind HTNV N protein. The 5′ end of the S-segment vRNA was observed to be necessary and sufficient for the binding reaction. Modeling of the 5′ end of the vRNA revealed a possible stem loop structure (SL) with a large single-stranded loop. We suggest that a specific interaction occurs between the N protein and sequences.

FASEB 2002

**Purification and Characterization of Hantaan Virus 246 kDa RNA Dependent RNA Polymerase (RdRp)**

Lisa Leon, William Severson, Mercy Thokola, Juan-Gabriel Gallegos, and Colleen Jonsson.
New Mexico State University, Department of Chemistry and Biochemistry.

Hantaviruses cause two serious illnesses in humans, hantavirus pulmonary syndrome (HPS) and hemorraghic fever with renal syndrome (HFRS). The negative-sense, tri-partite genome, segments—L, M and S—encode an RdRp, the G1 and G2 glycoproteins and the nucleocapsid protein, respectively. The RdRp, which has a molecular weight of 246 kDa, catalyzes the synthesis of the viral genomic, antigenomic and mRNAs required for the viral life cycle. However, no biochemical studies have been reported for any of these activities. The open reading frame of the Hantaan virus L-segment was cloned into the bacterial expression vector pET21b and expressed as a C-terminal hexahistidine fusion protein. At the N-terminal end, a T7 tag was incorporated for visualization by immunoblot. Expression of the RdRp, examined in six bacterial cell lines, was highest in Tuner(DE3) cells. Soluble RdRp was harvested from *E. coli* Tuner(DE3) cells, and partially purified. SDS-PAGE revealed a band between 150 and 250 kDa, which was confirmed on the corresponding immunoblot using anti-T7 tag antibodies.

Preliminary RdRp activity assays monitoring the incorporation of $\alpha^{32}$P-NTP using a primer-template RNA substrate have revealed the elongation activity of the RdRp *in vitro*.



ASV2002
Characterization of the RNA Binding Domain of the Hantaan Virus N Protein
Colleen B. Jonsson, William Severson, Xiaolin Xu, Nina Senutovitch and Samantha Claw
Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, NM 88003
A major role of the hantavirus nucleocapsid or N protein is to encapsidate the genomic and antigenomic viral RNAs. Only the genomic viral RNA, however, is packaged into the mature virion. We have defined a *cis*-acting element in the 5′ end of the vRNA that is preferentially recognized by the N protein. Further, we have recently mapped a specific region within the HTNV N protein, amino acids 175 to 217, that binds vRNA at a level similar to that of the full-length N protein. The HTNV N protein RBD does not resemble any of the currently published RBD motifs. We hypothesize that the sequence specificity of this interaction occurs through hydrogen bonding and electrostatic interactions between the protein side chains and base pairs in RNA grooves or disordered single-stranded regions. To identify the critical amino acids that create the HTNV N protein RNA binding motif, we have created 18 single amino acid mutations in basic amino and polar amino acids. The ability for each of these mutant proteins to bind the vRNA will be determined by filter binding analysis. Our long term goal is to build a comprehensive view of the general features of this unique RNA binding motif and its *cis*-acting element.

**Presentations**

| Date | Authors | Poster Title | Meeting Name & Location |
|---|---|---|---|
| 13-16 Jun | Juan Gallegos, William Severson, Colleen Jonsson | Purification and characterization of the catalytic core domain of the RDRP from Hantaan virus | 5th International Conference on HFRS, HPS and hantaviruses |
| 21-25 July | Lisa Leon, Juan Gallegos, William Severson, Colleen Jonsson | Purification and characterization of the catalytic core domain of the RDRP from Hantaan virus | American Society of Virology, WI |
| 13-16 Jun | Colleen Jonsson | Purification and characterization of the catalytic core domain of the RDRP from Hantaan virus. Jonsson | 5th International Conference on HFRS, HPS and hantaviruses, France |
| March 16, 2002 | Nina Senutovitch, Samantha Claw, William Severson, Colleen Jonsson | Encapsidation of the Hantaan viral RNA by its N protein Jonsson | NMSU Biology Symposium |
| March 16, 2002 | Lisa Leon, Mercy Thokala , William Severson and Colleen Jonsson | Characterization of the 246 kDa Hantaan RNA dependent RNA polymerase | NMSU Biology Symposium |
| April 23, 2002 | Lisa Leon, Mercy Thokala, William Severson and Colleen Jonsson | Characterization of the 246 kDa Hantaan RNA dependent RNA polymerase | FASEB; ASBMB, New Orleans, LA |

**Conclusions**.....

Because of the limited number of biochemical tools and reagents available for hantaviruses, our lab focused initially on establishing methods to purify soluble N protein (Jonsson *et al.*, 2001), and to measure its affinity for RNA (Severson *et al.*, 1999). This has allowed us to characterize the interaction of the N protein with viral and nonviral RNA, and led to the development of the first published biochemical model for encapsidation of the hantavirus genome (Severson, Xu, and Jonsson, 2001).

The approach we have taken to initiate studies into the HTNV RdRp has also required the establishment of methods to obtain purified RdRp and to assay its activity. Because of the large size of the RdRp, we anticipated that defining the conditions for expression and purification of the RdRp from bacterial systems would require an intensive effort. Therefore, we chose to complement these efforts and use the virion as a source of the RdRp as a means to develop enzymatic assays. These assays could then be used to characterize the RdRp, once purified from the recombinant system. Our preliminary results detailing these efforts are described in the following.

The future research aims to will establish methods to study the hantavirus polymerase as well as initiate studies to characterize the its substrate preferences. We have successfully initiated studies to express the RdRp, both as enzymatically active fragments and as a complete protein. These efforts are key to our long term goals of defining the enzymatic functions of the RdRp. Toward this goal, we have begun to establish conditions for RdRp activity by using infectious HTNV, and will apply those conditions to expressed RdRp to create an in vitro assay of enzyme function. Finally, in addition to the concrete results expected, our studies will provide novel and important information on the properties of hantaviruses during replication. Such information may serve as the basis for future investigations on other newly emerging viruses. Finally, this work will segue into a concerted effort that will lead to the design and screening of antiviral drugs to combat hantaviral diseases. Disrupting one or more functions of the RdRp is expected to result in effective disease treatment with little toxicity to host cells.

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# Purification and Characterization of the Sin Nombre Virus Nucleocapsid Protein Expressed in *Escherichia coli*

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**Sin Nombre virus is a member of the Hantavirus genus, family *Bunyaviridae*, and is an etiologic agent of hantavirus pulmonary syndrome. The hantavirus nucleocapsid (N) protein plays an important role in the encapsidation and assembly of the viral negative-sense genomic RNA. The Sin Nombre N protein was expressed as a C-terminal hexahistidine fusion in *Escherichia coli* and initially purified by nickel-affinity chromatography. We developed methods to extract the soluble fraction and to solubilize the remainder of the N protein using denaturants. Maximal expression of protein from native purification was observed after a 1.5-h induction with IPTG (2.4 mg/L). The zwitterionic detergent Chaps did not enhance the yield of native purifications, but increased the yield of protein obtained from insoluble purifications. Both soluble and insoluble materials, purified by nickel-affinity chromatography, were also subjected to Hi Trap SP Sepharose fast-flow (FF) chromatography. Both soluble and insoluble proteins had a similar $A_{280}$ profile on the Sepharose FF column, and both suggested the presence of a nucleic acid contaminant. The apparent dissociation constant of the N protein, purified by nickel-affinity and SP Sepharose FF chromatography, and the 5′ end of the viral S-segment genome were measured using a filter binding assay. The N protein–vRNA complex had an apparent dissociation constant of 140 nM.** 

Hantaviruses cause two illnesses in humans, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) (1). Hantaan virus (HTNV) and Sin Nombre virus (SNV) are the major causative agents of HFRS and HCPS, respectively. Hantaviruses have a negative-sense, single-stranded RNA genome that consists of three segments, S, M, and L (2, 3). Transcription of each vRNA by the viral-encoded RNA-dependent RNA polymerase (RdRp) yields a complementary RNA (cRNA), which in turn is used as a template to generate vRNA by the RdRp. In contrast to the hantaviral mRNAs, which do not associate with the viral nucleocapsid protein (N) (4), the vRNA and cRNA replicative templates are encapsidated by N. The interactions of the hantaviral RNAs and N are not well defined. Gott *et al.* showed the presence of a nonspecific RNA binding domain in the C-terminus of the HTNV N protein (5). In another study, the HTNV N bound preferentially to the viral genome rather than to nonviral RNA (6). More extensive biochemical studies of RNA–protein or protein–protein interactions will require sufficient quantities of highly purified, soluble, monodispersed N protein that are free of contaminating ribonucleases, nucleic acids, and RNA binding proteins. Toward this goal, we have investigated methods to recover hantaviral N from a bacterial expression system in a form suitable for biochemical analysis.

Our previous purification strategy, as well as that of others, relied on using denaturing methods to recover soluble expressed N from bacteria (5, 6). Because renaturing does not always result in native protein, large losses in functional N are common. Comparing yields of HTNV and SNV N after denaturing and renaturing

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shows that the SNV N protein is more readily recovered and probably is more stable (6). No obvious reason for the difference in ease of recovery of soluble SNV N versus HTNV N can be deduced from examining the amino acid sequences of the two proteins. The most notable difference is the substitution of seven Gly residues in the HTNV N protein with Asn, Ala, Ser, or Asp in the SNV N protein. All of these substituted amino acids are more hydrophilic than Gly (7), and if they are located on the surface of the protein, they could facilitate the interaction of the protein with an aqueous environment. Insight into the molecular basis for the differences awaits a three-dimensional structure that could provide information on the solvent accessibility of these residues.

Herein, we report studies leading to the development of a rapid soluble extraction protocol and chromatography method for recovery and purification of the SNV N protein expressed in *Escherichia coli*. We further test this method for suitability for preparing N proteins of three other hantaviruses.

## MATERIALS AND METHODS

### *Reagents and Expression Vectors*

Restriction enzymes and Vent polymerase were purchased from New England Biolabs (Beverly, MA). T4 DNA ligase and kinase were purchased from Gibco BRL (Grand Island, NY). All chemicals were purchased from Sigma (St. Louis, MO). The plasmid expression vectors pSEO-N pET-1 and pPUU-N pET-1 (gift from Brian Hjelle, M.D., University of New Mexico) contain the open reading frames (ORF) for the Seoul virus 80/39 (SEOV) and Puumala virus P360 (PUUV) N proteins (8). HTNV N protein was expressed from pHTNV-N as described previously (6).

### *Construction of the SNV N Protein Expression System*

SNV, strain CC107, S-ORF was amplified by PCR from SNVS/pCRII (9) with two DNA oligonucleotide primers, SNV S-Nhe, 5′-TCACTGGATTCCATATGGCTAGCACCTCAAAGAATGC, and SNV S-Xho, 5′-TCACTGGATTCTTACTCGAGAAGCTTAAGTGGTTCCTGGTTAGAAATTTC. Primers were synthesized with an ABI 394 DNA/RNA synthesizer. The SNV S-5′ ORF contained a *Nhe*I restriction enzyme digestion site at the 5′ end (underlined) and the SNV-3′ ORF contained a *Xho*I digestion site at the 5′ end (underlined). The PCR product and the pET21b expression vector were digested with *Nhe*I and *Xho*I (New England Biolabs). The products were separated by agarose electrophoresis and purified with the QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol. The SNVS-ORF and pET21b were ligated with T4 DNA ligase (Gibco BRL). Clones of SNVN/pET21b were selected by restriction enzyme mapping and confirmed through DNA sequencing using the LiCOR 4200 IR$^2$ automated sequencer. SNVN/pET21b was transformed into competent *E. coli* BL21(DE3) cells (Novagen) for expression studies.

### *Expression and Purification of SNV N Protein*

In general, *E. coli* BL21(DE3) cells harboring the SNVN/pET21b and other hantavirus N proteins were grown overnight in 200 ml of Luria–Bertani (LB) medium containing 200 $\mu$g/ml ampicillin. After 16–19 h, cells were diluted 1:20 in LB medium containing 200 $\mu$g/ml ampicillin and grown for 1 h at 30°C at which time isopropylthiogalactoside (IPTG) was added to a final concentration of 0.6–0.8 mM to induce expression of the protein. The methods that follow are based on a harvest of an 800-ml bacterial culture grown in a 2-liter flask. Soluble and insoluble extractions were performed and subjected to both metal chelate-affinity chromatography and ion-exchange chromatography. In general, fractions were collected and 20 $\mu$l of each fraction was examined for SNV N protein by SDS–PAGE or separation by 4–12% NuPAGE Novex Bis-Tris Gels (Invitrogen) and Western blot analysis. Western blots were performed using polyclonal rabbit sera to SNV N (diluted 1:4000) or mouse sera to polyhistidine (diluted 1:2000) (Sigma; monoclonal clone His-1). The secondary antibodies were alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG (diluted 1:1000) (Promega), which were developed using Western Blue Reagent (Promega). In addition, protein concentrations were measured using the Bradford method (10) with Bio-Rad Micro-Assay reagents as recommended.

### *Purification Using Nickel Nitriloacetate Agarose under Native Conditions*

After induction with IPTG for 1.5–5 h, 800 ml of the culture were harvested and resuspended in 50 ml of ice-cold solubilization buffer [50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 20 mM $\beta$-mercaptoethanol, protease cocktail inhibitor (Boehringer Mannheim), and 0.50 mg lysozyme with or without Chaps (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate)]. The suspension was Dounce homogenized for 30 min on ice. Following lysis, the material was sonicated five or six times on ice, with a cycle consisting of 1 min on and 2 min off, in a Branson Sonifier until the material clarified. Soluble and insoluble materials were separated by centrifugation at 30,000*g* for 1 h. Insoluble material was saved and placed at −80°C for subsequent extraction. To the soluble fraction, 1 ml of a 50% suspension of nickel nitriloacetate agarose (Qiagen, Chatsworth, CA) was added per liter of starting material and stirred at 4°C for 2 h

to overnight. The material was added to a column and washed with 10 column volumes of wash buffer [50–100 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole, 20 mM $\beta$-mercaptoethanol (BME)]. The N protein was eluted from the resin with elution buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 250 mM imidazole, 20 mM $\beta$-mercaptoethanol). Fractions were either stored at −80°C or directly placed into 500 ml of dialysis buffer [40 M Hepes, pH 8.0, 200 mM NaCl, 0.1 mM dithiothreitol (DTT)] for 2–4 h with two changes of buffer.

*Purification Using Nickel Nitriloacetate Agarose under Nonnative Conditions*

Insoluble material was resuspended on ice in 50 ml denaturing buffer (DB; 50 mM sodium phosphate buffer, pH 8.0, 0.5 M NaCl, 20 mM BME, 8 M urea, with or without 10 mM Chaps). The suspension was Dounce homogenized and sonicated as described above. The extract was gently shaken at room temperature for an additional 30 min and then centrifuged at 30,000*g* for 1 h. The supernatant was applied to a 1-ml column preequilibrated with DB, pH 8.0. The column was washed with 10 column volumes of DB, pH 8.0; DB, pH 6.3; and DB, pH 5.9. The N protein was eluted with 10 column volumes of DB, pH 4.5. One-milliliter fractions were collected and 10 $\mu$l of each fraction was examined for SNV N protein by SDS–PAGE and Western blot analysis. Fractions containing N were dialyzed over a 5-day period into a final buffer with 20 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 500 mM NaCl, 5% glycerol, with urea concentrations decreasing daily (day 1—4.0 M, day 2—2.0 M, day 3—1.0 M, day 4—0.5 M, day 5—0 M).

*Purification of SNV N Protein under Native Conditions with SP Sepharose Chromatography*

Material isolated from soluble or insoluble/refolded fractions was subjected to an overnight dialysis into 50 mM Mes, pH 6.2, 200 mM NaCl. Fifty milliliters of the dialyzed material was then bound to a 1-ml Pharmacia Hi Trap SP Sepharose fast-flow column preequilibrated with 50 mM Mes, pH 6.2, 200 mM NaCl at a flow rate of 0.5 min/ml. A 10-ml gradient from 200 mM to 1 M NaCl was run by the FPLC at a flow rate of 1.0 ml/min. One-milliliter fractions were collected and 20 $\mu$l of each fraction was examined for SNV N protein by SDS–PAGE and Western blot analysis as described above.

*Filter Binding Assay of SNV N Protein*

The filter binding assays were as described previously (6) using synthetic RNA and purified N protein. The oligoribonucleotide corresponding to the 5′ end of the SNV S-segment vRNA (5′-UAgUAgUAgACACCU-UgAAAAgCAAUCAAgAAUUUACUU-3′) was synthesized and HPLC purified by Integrated DNA Technologies, Inc. (Coralville, IA). Synthesis was performed on a 1 $\mu$M scale. The synthetic RNA was labeled at the 5′ terminus with [($\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase (New England Biolabs) and purified on Quick Spin columns (Roche). SNV N protein was serially diluted in binding buffer (40 mM Hepes, pH 7.4, 100 mM NaCl, and 5% glycerol) to give a final concentration range of $5.6 \times 10^{-9}$ to $5.6 \times 10^{-6}$ M. Apparent dissociation constants ($K_d$) were calculated by fitting a nonlinear binding curve to the empirical data using the Origin program (MicroCal). The apparent $K_d$ corresponds to the concentration of N protein required to obtain half-saturation; assuming the complex obeys a simple binding bimolecular equilibrium. We assumed the plateau in the percentage binding of the RNA represents complete binding of the RNA, to allow the calculation at half-saturation.

*Dynamic Light Scattering of SNV N Protein*

SNV N protein was examined by a DynaPro LSR (Protein Solutions, Inc.) to determine its molecular weight. Measurements were made in the buffers in which the proteins were eluted from the column.

## RESULTS AND DISCUSSION

*Optimization of Soluble Extraction and Purification Conditions for the SNV N Protein*

Small-scale (800 ml) bacterial cultures were used to qualitatively define the optimal extraction and nickel-affinity purification conditions for the SNV N protein. Three extraction/running buffers were examined: buffer 1—100 mM sodium phosphate, pH 8.0, 300 mM NaCl, 30 mM imidazole; buffer 2—100 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 5% glycerol; buffer 3—100 mM sodium phosphate, pH 8.0, 1 M NaCl, 10 mM imidazole, 5% glycerol. Buffer 1 resulted in the greatest yield of soluble SNV N protein. Comparison of buffer 1 with an identical buffer with 10 mM imidazole showed that the higher concentration of imidazole did not reduce background binding of nonspecific proteins; therefore, the imidazole was lowered to 10 mM in subsequent experiments.

*Purification of the SNV N Protein Following IPTG Induction*

Induction periods of 1.5 or 5.0 h were tested for influence on SNV N protein yield from 3200-ml cultures of bacteria. N protein was extracted and purified by nickel-affinity chromatography under native conditions as described under Materials and Methods. Fractions

from each of the purifications were examined by Coomassie blue staining (Figs. 1A and 1B) as well as by Western blot analysis (data not shown). The N protein had an apparent molecular weight slightly greater than its predicted molecular weight of 49 kDa (11). The total yield of protein in fractions 1–4 from the 1.5-h induction was 7.6 mg, or 2.4 mg/L, while the yield from the 5-h induction was 3.3 mg, or 1.0 mg/L (Table 1). Thus approximately twice as much total protein was recovered following the shorter induction period.

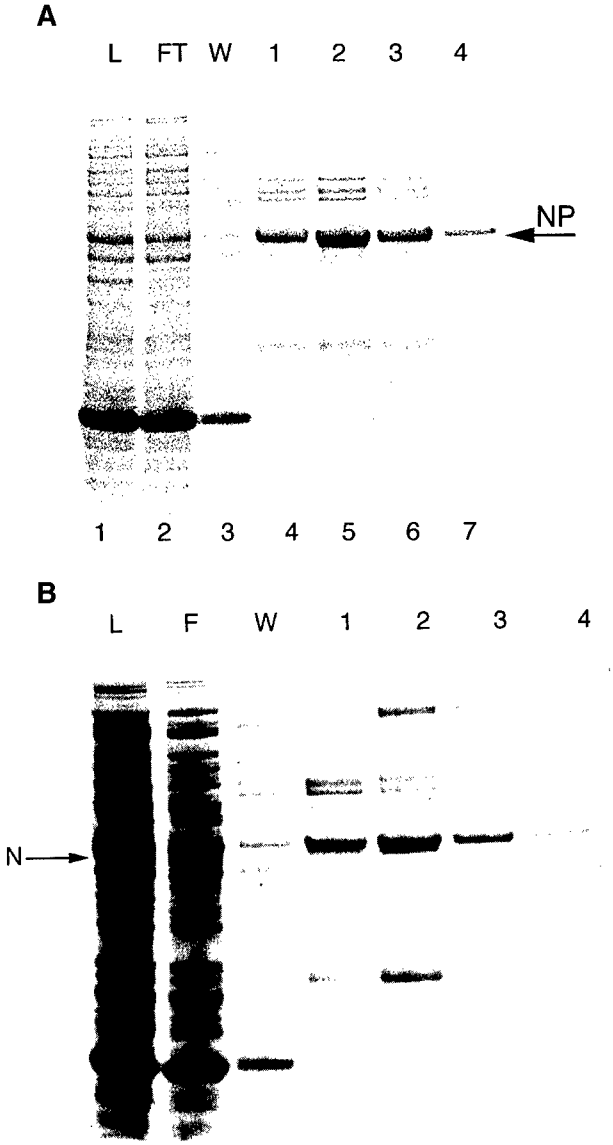


**FIG. 1.** Influence of IPTG induction time on SNV N yield. 3.2-L cultures of bacteria expressing SNV N were extracted at 1.5 (A) or 5.0 h (B) after adding IPTG. Proteins were extracted with Chaps and purified by nickel-affinity chromatography and analyzed by electrophoresis on 4–12% Bis-Tris gels and Coomassie blue staining. L, column load; F, column flowthrough; W, column wash; 1–4, fractions eluted from column. An arrow points to the N protein.

**TABLE 1**

Summary of Ni-NTA Purification Yields for Various N Proteins

| Hantaviral N protein | Induction (h) | Yield (mg/L) | Chaps added | Extraction method[a] |
|---|---|---|---|---|
| SNV | 1.5 | 2.4 | No | N |
| | 1.5 | 2.4 | Yes | N |
| | 5.0 | 1.0 | No | N |
| | 1.5 | 0.09 | Yes | D |
| | 1.5 | 0.07 | No | D |
| SEOV | 1.5 | 0.45 | Yes | N |
| PUUV | 1.5 | 1.3 | Yes | N |
| HTNV | 1.5 | ND | Yes | N |

*Note.* ND, not determined.
[a] N, native; D, denaturing.

### *Expression and Purification of the SNV N Protein in the Absence of Chaps*

The zwitterionic detergent, Chaps, has been frequently used to enhance the solubility of proteins recovered during both native and denaturing extraction protocols (12). Thus, our initial extraction and running buffers included this detergent. To determine if Chaps enhanced the solubility and yield of the SNV N protein, we extracted bacterial cells and performed nickel chromatography using the same native conditions as described above for the time-course study, but without the addition of Chaps. Fractions from each of the purifications were examined by Coomassie blue staining (Fig. 2) as well as by Western blot analysis (data not shown). The total yield of protein obtained in fractions 1 through 4 was 7.7 mg (2.4 mg/L). This yield was similar to that observed when Chaps was included in the extraction buffer (Table 1), although inclusion of Chaps resulted in a higher proportion of the total protein in fraction 2 of the elutant (Figs. 1A and 1B). In contrast to these results, inclusion of 10 mM Chaps in the denaturing protocol did enhance SNV N recovery (Table 1 and Figs. 3A and 3B). Overall, the native extraction protocol, irrespective of the inclusion of Chaps, yielded a greater amount of N protein than the denaturing methods; however, the denaturing protocol (Figs. 3A and 3B) yielded a much more homogeneous and pure preparation of N than the native extraction (Figs. 1 and 2).

### *Expression and Purification of the Other Hantavirus N Proteins Using Methods Developed for the SNV N*

In previous studies, we were unable to purify HTNV N protein expressed in *E. coli* using native conditions, although we could recover some protein (approximately 1.5 mg per liter of culture) from the insoluble fraction using denaturing conditions (6, 12).

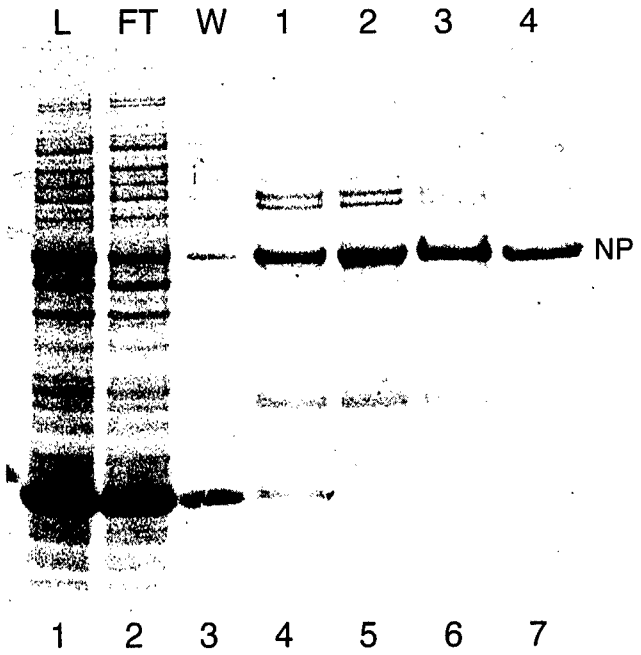


**FIG. 2.** Native purification of the SNV N protein by Ni-NTA chromatography without Chaps. A representative purification from cellular material extracted without Chaps is shown. L, column load; F, column flowthrough; W, column wash; 1–4, fractions eluted from column. These samples were subjected to separation by 4–12% NuPAGE Novex Bis-Tris gels.

To determine if the methods that we devised for extraction and purification of soluble SNV N would be applicable to other hantavirus N proteins expressed in *E. coli*, we tested them for recovery of expressed N of three hantaviruses that cause hemorrhagic fever with renal syndrome: HTNV, PUUV, and SEOV. Proteins were extracted with the nondenaturing buffer containing Chaps and isolated by nickel-affinity chromatography. Eluted fractions were separated by electrophoresis on gradient gels and visualized by Coomassie blue staining (Figs. 4A–4C). The total yield of PUUV or SEOV V N proteins from 1 liter of culture was 1.3 or 0.45 mg/L, respectively (Table 1). The yield of HTNV protein was difficult to compare to the other N proteins because of higher background; nevertheless, the amount visualized by Coomassie blue staining was much greater than we previously observed using denaturing and refolding methods.

### *Purification of the SNV N Protein by SP Sepharose Fast-Flow Chromatography*

To further refine our purification protocol for SNV N, we subjected material recovered by nickel-affinity chromatography from both the soluble and the insoluble refolded fractions to chromatography on a 1-ml Pharmacia SP Sepharose FPLC column with a gradient of 200 mM to 1 M NaCl (Fig. 5A). Before being loaded onto the FPLC column, the nickel-affinity column fractions were pooled and dialyzed overnight against 50 mM Mes, pH 6.2, 200 mM NaCl. This dialysis step enhanced the binding of the protein on the SP Sepharose matrix. From both the native and the refolded protein preparations, two peaks were evident in the FPLC column $A_{280}$ profile. Figures 5A and 5B show the profiles for the N protein recovered following refolding. Examination of the proteins in those peaks by SDS–PAGE revealed little N protein in the first peak (Fig. 5B, lanes 1 and 2). The $A_{260}$:$A_{280}$ ratio of this peak was 1.7, which strongly suggests the presence of nucleic acids in these fractions. The second peak, which eluted at 9 min in 660 mM NaCl, had most of the N protein (in both refolded and soluble preparations) (Fig. 5B, lanes 3 and 4). These data suggest that the refolded material has properties similar to the native material. At present, we estimate that the isolated N protein has 85% or greater homogeneity. A summary of the yields from the NTA and SP Sepharose FF is presented in Table 2.

Light-scattering analysis of N protein showed that the N proteins isolated from the nickel-affinity resin and the SP Sepharose were 96 and 87% monodispersed, respectively (data not shown). As reflected in the results

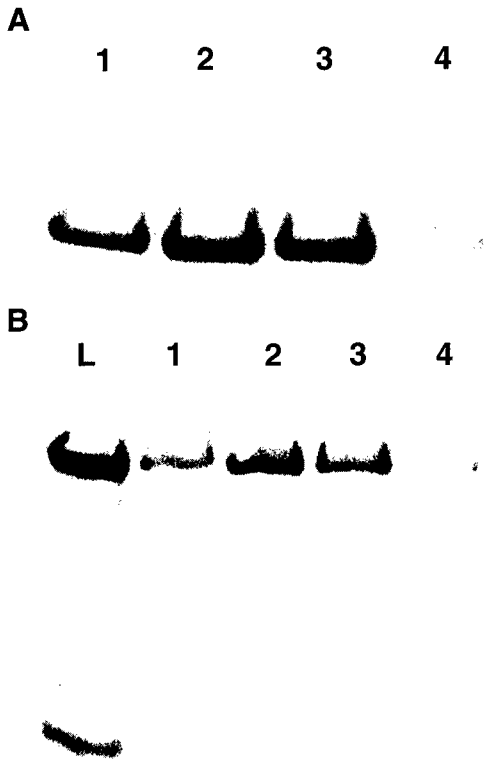


**FIG. 3.** Denaturing purification of the SNV N protein in the presence and absence of Chaps by NTA chromatography. Purifications are shown from insoluble cellular material extracted with Chaps (A) and without Chaps (B). L, column load; 1–4, fractions eluted from column. These samples were subjected to separation by 4–12% NuPAGE Novex Bis-Tris gels.

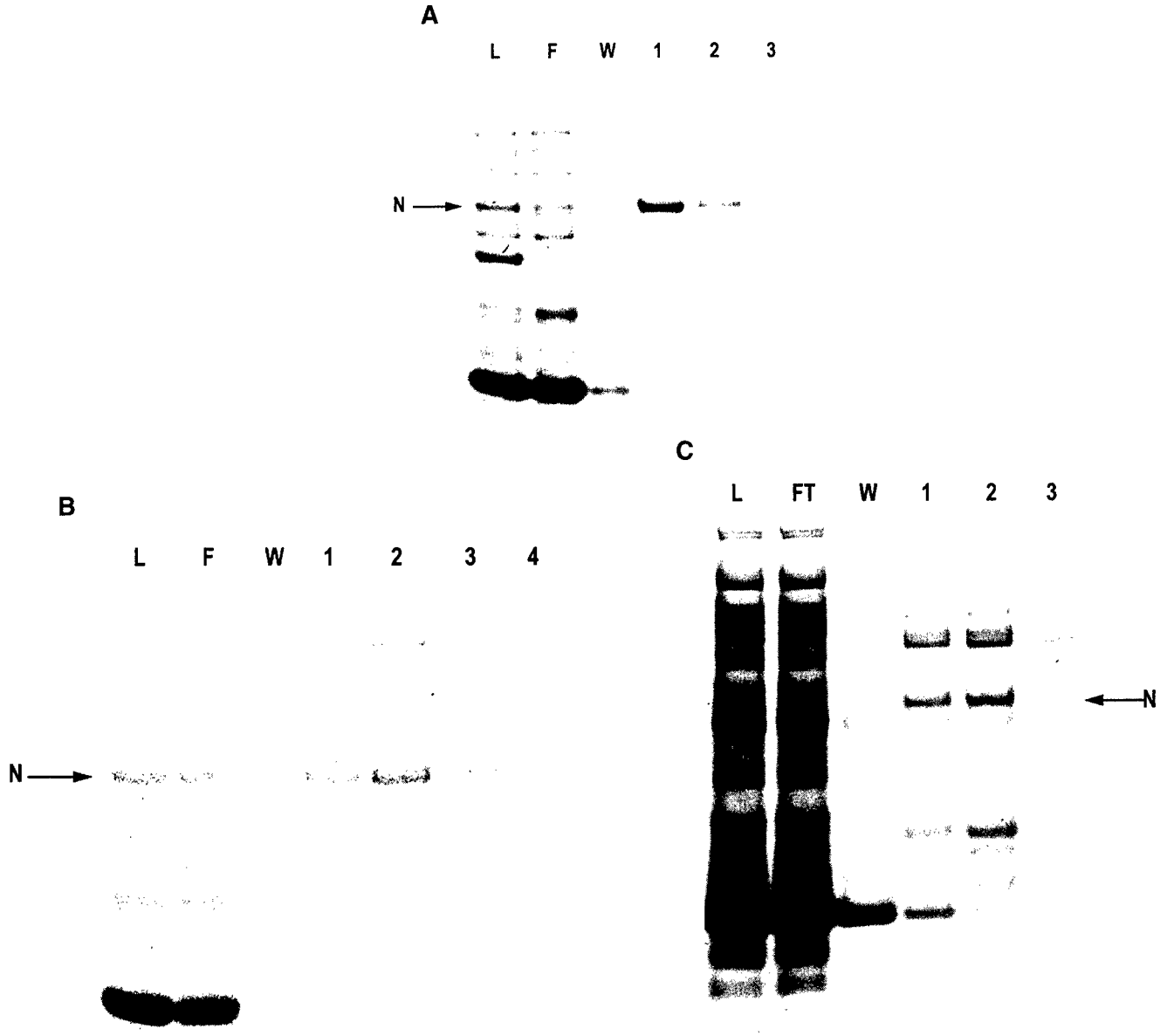


**FIG. 4.** Purification of PUUV, SEOV, and HTNV N protein by NTA chromatography. Purifications are shown for cellular material extracted with Chaps for PUUV (A) and SEOV (B) and HTNV N protein (C). L, column load; F, column flowthrough; W, column wash; 1–4, fractions eluted from column. An arrow points to the N protein. These samples were subjected to separation by 4–12% NuPAGE Novex Bis-Tris gels.

stated above, the molecular mass of the N protein isolated by the nickel-affinity resin, 87 kDa, was greater than the predicted molecular mass, 49 kDa, and supports the presence of a nucleic acid contaminant. Light scattering showed that the N protein isolated by SP Sepharose chromatography (Fig. 5B, lanes 3 and 4) had a molecular weight of 54 kDa.

*RNA Binding Activity of the SNV N Protein*

To determine if the extraction and purification protocol that we devised resulted in SNV N protein suitable for biochemical studies, we tested the purified protein in an RNA binding assay. Based on earlier findings suggesting that the terminal nucleotides of the hantaviral S-segment are involved in encapsidation and nucleocapsid assembly (13), we prepared a 39-base synthetic oligoribonucleotide for the binding assay. This oligonucleotide, designated SNV-vRNA 1-39, corresponds to the 5′ end of the nascent S-segment vRNA. Filter binding experiments performed with increasing concentrations of purified SNV N protein and a constant amount of SNV vRNA were used to generate a binding isotherm. The apparent dissociation constant ($K_d$), calculated as half-maximum binding, was approximately 140 ± 30

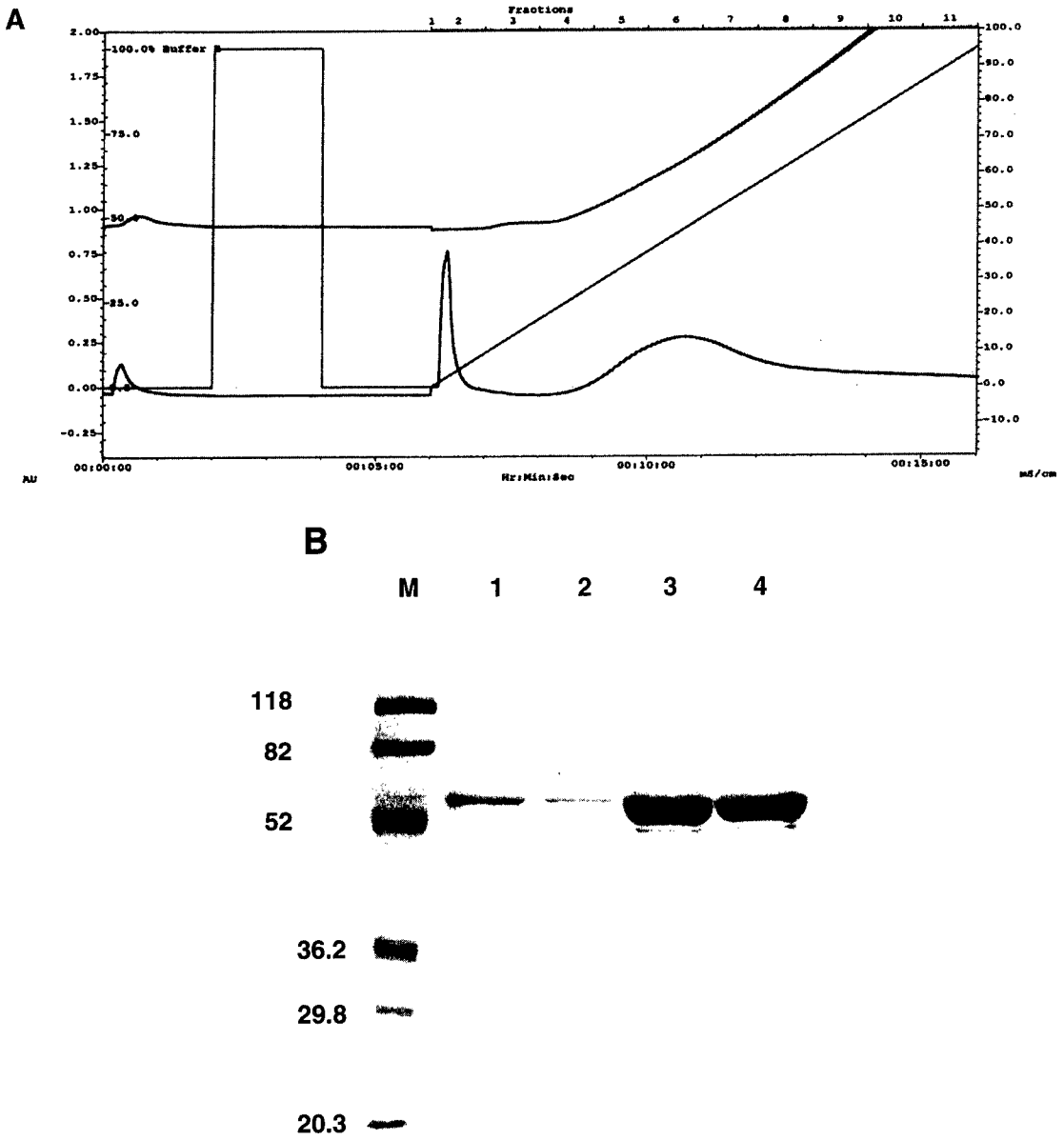


**FIG. 5.** SP Sepharose chromatography of SNV N protein. (A) 100 ml of refolded N protein was loaded onto a 1-ml Pharmacia SP Sepharose column. The column profile shown was run from 200 mM to 1 M NaCl. (B) Fractions from the two major peaks shown in the chromatogram in (A) are presented: Peak 1, fractions 1 and 2 (lanes 1 and 2); peak 2, fractions 5 and 6 (lanes 3 and 4). These samples were subjected to separation by 12% SDS–PAGE.

nM. This interaction shows a similar binding affinity reported for the HTNV N-protein vRNA (1-39) complex (132 ± 9) (13). We suggest that this specific oligoribonucleotide substrate competed for N protein binding, thereby displacing any remaining nucleic acid contaminant. These data are further supported by recent competition experiments with the HNTV N protein (13). These data indicate that the purified SNV N protein is of sufficient quality and purity to substitute for authentic viral protein in filter binding assays.

**TABLE 2**

Soluble Purification of the SNV N Protein

| Step | Volume (ml) | Total protein (mg) | Estimated purity (%) |
|---|---|---|---|
| Culture medium | 3200 | ND | ND |
| NTA | 20 | 3.4 | ≈70% |
| SP Sepharose | 3 | 1.8 | >85% |

*Note.* ND, not determined.

## ACKNOWLEDGMENTS

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